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Genetics of resistance to Bacterial inhibitors

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GENETICS OF RESISTANCE TO BACTERIAL INHIBITORS.

I. - INTRODUCTION.

The development of new antibacterial agents has brought to the foreground a new aspect of evolution in microorganisms, viz. drug adaptation. The practical importance of this phenomenon is so great, that much study has been dedicated to the problem of its origin, at a time when genetic analysis of microorganisms is in its infancy and the prejudice that their genetic systems are thoroughly different from those of the more fully investigated higher organisms is still widespread. Thus it is perhaps not too surprising, that the issue has from the beginning been a highly controversial one, and that the discussion has been centered around motives which are essentially similar to those prevailing in the last century on theories of evolution in general.

It may be added that present knowledge allows one to discriminate much of the current prejudice on microbial genetics and thus to settle a fairly large portion of the controversy on drug adaptation.

Most microorganisms can, in fact, show at least some degree of adaptation to most of the drugs, to whose action they are subjected. Rarely adaptation reaches a very high degree (i.e., to high concentration of the drug) after one or few exposures to the drug of a bacterial population. More commonly, adaptation develops gradually, but even so it may often result, at the end of a long process, in a high level of resistance being attained in the adapted strain. Whether adaptation occurs gra-

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dually or suddenly depends mostly on the drug, and presumably therefore on the mechanisms of its action; too little of which is usually known, however, to permit further speculation along these lines. Complete single step adaptation is observed with streptomycin and, perhaps less throughly, with isoniazide; gradual adaptation was first described in genetical terms for penicillin by Demerec (1945, 1949) and hence called by this author the «penicillin pattern» of drug-adaptation as contrasted with the «streptomycin pattern».

Another angle from which the facts concerning drug-resistance can be viewed is the variability of the resistance level gained by a given type of microorganisms in a single exposure to the drug. Survivors of a bacterial population plated in the presence of a suitable concentration of the drug may show, on successive subculture, an increased resistance, as compared with that of the original strain; the measure of the resistance level thus acquired by one exposure (often called first-step-resistance) may show differences between independent isolates. The variability of first-step-resistance is of course likely to be highest for drugs of the streptomycin pattern, these being defined as permitting full resistance to be developed with the first step. However, a variation of the degree of first-step-resistance is observed, even if less impressively, for drugs to which only gradual adaptation is possible, and, apart from variability of the level of resistance acquired, morphological, biochemical, genetical variation can be detected among first-step-resistants to a given drug. Only with one agent, and this perhaps because of insufficient analysis, is no important variability of first-step-resistance encountered, namely azide-resistance in *E. coli*.

A technical detail of some importance is whether drug adaptation is carried out in solid or in liquid media, containing the drug at suitable concentrations. Adaptation in liquid medium for drugs of the «penicillin pattern», by means of successive subcultures in increasingly higher concentrations of the drug may result in the bacterial population showing an almost continuous rise in the resistance level, although stepwise increases are also observed. Increase in steps is forcefully observed when adaptation to such drugs is carried out in solid media, since every successive transfer on a drug-agar plate with a suitably high inoculum and drug concentration is likely to give rise to a definite increase in resistance of some of the survivors.

Finally, observations may bear on the stability of resistance thus acquired, and here a full range of stability degrees may be observed, though carefully isolated resistant survivors which show some degree of

resistance on a successive subculture tend to keep this resistance more or less unaltered over a number of transfers.

This oversimplified summary of the main facts may serve as an introduction to the explanation which have been offered for the behaviour exhibited by microorganisms in the presence of antimicrobial drugs.

First let us consider the explanation which is provided by standard genetical theory for adaptive changes in the reaction of an individual or a population to a given environment. Two mechanisms which are not mutually exclusive may come into play:

A) *Physiological adaptation*: every individual of a given genotype can adapt to a certain range of environmental variation. The new environment often directly induces the changes, as in enzyme adaptation; on return to the old environment the change usually disappears both rapidly and entirely. Adaptation and deadaptation leave the hereditary background unaltered even if carried out repeatedly.

B) *Genetic adaptation*. Individuals of a given genotype are unable to cope effectively with the situation created by a new environment. Mutation or other genetic changes provide the basis of new heritable variation. If one or more mutants can propagate themselves in the new environment, they will initiate clones which will eventually be substituted for the old population. Thus the environment acts by selecting fitter variants, endowed with a hereditary capacity to withstand the new conditions. Genetic adaptation is essentially preadaptation, in contrast with the physiological kind (A) which is postadaptive. The change is not readily reversible as in the preceding instance, because reversion can occur only by back mutation or mutation at another locus with modifying effects on the primary mutation, and these kinds of genetic changes prove to be generally very rare.

These two mechanisms of adaptive change have been fairly well ascertained in a number of instances and some models have been examined in detail (Monod, 1952; Ryan, 1952, to quote an example of each type). Formal genetic analysis can ascertain which of these two mechanisms operates by an analysis of individual behaviour from homogeneous populations, i.e. essentially clones of cells. Clones of sufficient size for mutation to have occurred in one or more of its members will form an incipiently heterogeneous population on which selection may act. Deliberate mixture of genetically different lines will provide means of analysis by genetic transfer in order to analyse the genetic nature of the heritable changes. The effort of the geneticist may therefore be directed to the detection of the relative importance of the two mechanisms in the deter-

mination of drug-resistance, i.e. to the control of pre- and post-adaptive changes, by the use of such techniques.

A variety of alternative theories of drug adaptation have been proposed; just to summarise the most important ones:

C) Direct induction of genetic change by the drug (Linz 1950, others). The change to resistance is a truly genetic one, i.e. heritable etc. but is induced by the drug itself, and is hence of a postadaptive nature, which has no parallel in the above scheme.

D) Self-adjustment of enzyme-balances to permit growth in the new conditions without nuclear change or selection (Hinshelwood 1946). Although this theory is not substantially different from what has been called physiological adaptation, this author has pushed its application so far as to cancel or minimise the role of selection or mutation. It may be noted that this expectation may represent an oversimplification of the genetic theory, as it tends to draw no distinction between the individual and a population. This theory could therefore be formulated in terms which would include the preadaptation theory. If the population is studied as a whole, and no attempt made to resolve its elements, the enzyme balance of the mass will doubtless be found in the long run to show adaptive adjustments of the kind subsumed under this hypothesis. For an understanding of the genetic basis of such changes, however, we must examine the adaptive responses of individual cells, and we shall find that the best studied cases of apparently directed, post adaptive changes of whole populations can be interpreted in terms of pre-adaptive changes of small numbers of its constituent cells.

Both these theories demand a post-adaptive change and ignore preadaptation. An attempt to compromise was recently advanced by Yudkin, under the name of «clonal variation with selection», where it is (implicitly) assumed that phenotype and genotype of a cell are identical, and variation originates from the distribution in unequal parts at cell division of the systems responsible for resistance. Actual cell resistance determines the average resistance of the progeny (phenotype = genotype); selection acts in the same way as in genetic adaptation. Here changes would be preadaptive. It may be added already at this stage that the knowledge on bacterial genetics is strongly at variance with such assumptions. Again, however, Yudkin's approach may be considered as an aversimplification of the genetic theory, assuming an inordinately extreme polygenic picture.

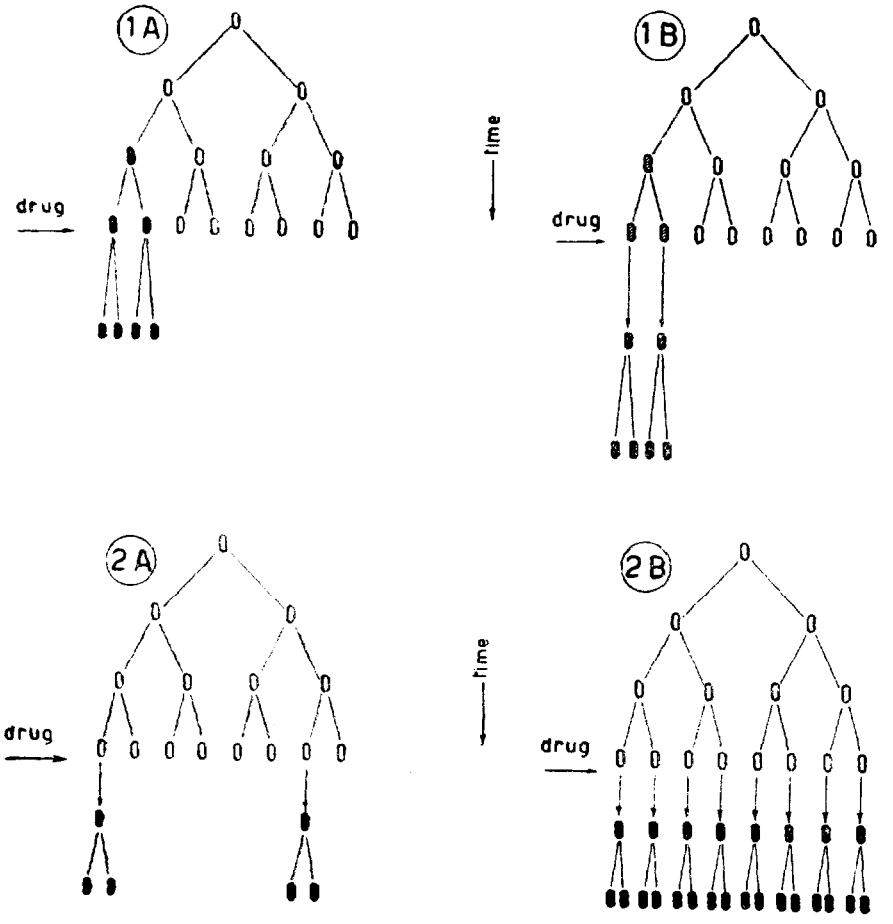

Fig. 1. - Models of drug resistance. Explanation in text.

II. - THE ANALYSIS OF INDIVIDUAL CHANGES.

A visual aid to the formal analysis of the various interpretations is given in figure 1. We can be satisfied, at this stage, with the analysis of two, or better two pairs of models taken to explain the origin of drug resistance. The first (1 A) shows strict genetic preadaptation. The change occurs prior to addition of the drug, and is also phenotypically expressed before it. The second model (1 B) differs from the first in that mutation determines only the potential capacity to withstand the drug; the necessary enzymatic systems will however, be manufactured or adjusted only in the presence of the antibacterial agent. Here, genetic adaptation is coupled with physiological adaptation.

The third and fourth models (2 A and 2 B) show two varieties of «physiological adaptation» with low and high survival of the mass of the population respectively. In the third scheme, each cell has the same probability of giving rise to a resistant clone following the addition of the drug, or if the probabilities differ from cell to cell, there is no simple relationship between the probability of survival of different cells and their common ancestry. In both models however, it is assumed that heritable resistance is postadaptive.

The essential difference between models 1 A, 1 B on one side and 2 A, 2 B on the other is whether or not preadaptation plays a role. If it does, two verifiable consequences follow from this: 1) resistants will appear in clones; 2) it will be possible to accomplish selection of resistant mutants in the absence of the drug. Both these methods have been used experimentally, with results to be summarised now. It may be added that the difference between schemes 1 A and 1 B will demand physiological investigations subsidiary to the major issue; and that even if the difference between models 2 A and 2 B, as well as between 2 B and the remainder may seem obvious on the basis of the ratio of output of resistants to input of sensitives, the analysis of this ratio may demand a more careful scrutiny than one may on first sight be led to believe. Thus situations in which even 100% of the cells seem to adapt may be entirely deceptive, as we shall see later, with incompletely bacteriostatic drugs, or in other conditions. If it is ascertained that this ratio is low, as is usually the case, discussion may be confined to distinguishing between models 1 A (or 1 B) and 2 A.

Clonal appearance of resistant variants.

Pedigree analysis by isolation of cells after each division has been attempted by Zelle (1942) in the case of the smooth to rough variation

in *Salmonella typhimurium* and showed that the mutants appeared in clones. Unfortunately there is a complete bar to this direct approach in drug-resistance of bacteria, owing to the extremely low frequency of mutation for resistance, so that millions of isolation of cells immediately after division would be necessary. It is not surprising, therefore, if that various statistical devices have been elaborated to meet this deadlock.

1) A detailed analysis was first given by Luria and Delbrück (1943). They considered that, if there is preadaptation and therefore resistant mutants are to be found in clones, the number of resistant mutants in parallel cultures each started from a single sensitive cell and grown under identical conditions should be highly variable and their distribution should indeed be predictable, given certain easy assumptions on the probability of mutation per cell, and the growth rate and lag of mutants.

The method was extensively used by a number of authors in a crude way, by comparing the variance of observed numbers of resistant mutants in parallel cultures with that expected under model 2 B, assuming this to give rise to a Poisson distribution, as would indeed be the case if, in model 2 B, every cell had the same probability of adapting, independently of the ancestral history of the cell. In a Poisson distribution the variance should be equal to the mean; in all but three series of experimental data observed variances of numbers of resistant mutants have instead been much higher than the respective means. This was taken to imply that model 2 B was untenable, and therefore to be discarded in favor of models 1 A or 1 B. This crude analysis is often referred to under the name of «fluctuation test». The three exceptions quoted are: streptomycin dependence in *E. coli* (Newcombe and Hawirko 1949) where clones of dependents obviously cannot form in absence of the drug, so that the test is inapplicable; streptomycin-resistance in Staph. aureus observed by Welsch (1952), who also showed the existence of a strong selection against the resistant mutants, so that this case approaches that of streptomycin-dependence; finally, penicillin-resistance in Staphylococcus aureus (Eriksen 1949) where among other difficulties the use of small samples from the cultures made the statistical tests inefficient. These exceptions cannot therefore be taken as proofs of model 2 A.

It must be stressed that the analysis offered by the socalled «fluctuation test» is open to two sources of error. One, which was pointed out by Eriksen (1949) and by Dean and Hinshelwood (1952), is based on the fact that no check is available of uncontrollable differences of en-

vironmental origin between parallel cultures. We shall see later how this objection can be met.

For a more satisfactory support of model 1 (A or B) a comparison of the observed distribution of resistant colonies with that expected under one of these models would therefore seem essential. The mathematical theory was provided by Lea and Coulson (1949), and extended by Armitage (1952) to cover certain necessary side assumptions (phenotypic delay, back mutation, unequal growth rate of mutant and parent). Analysis of data published by Luria and Delbrück, and by Newcombe (1948) on phage resistance showed a tolerably good fit with the theory; a good fit was obtained by Ryan (1952) for mutation to lactose-adaptability (model 2 B) in *E. coli*. As to drug-resistance proper, only one example (Newcombe and Hawirko, 1949, streptomycin-resistance in *E. coli*) was given, and showed a good fit with model 1.

2) A method, which may be called the method of *statistical clones* was proposed by Newcombe (1949). It consists of the comparison between the number of resistant colonies arising from plates on which bacteria have been allowed to grow into microcolonies prior to the application of the antibacterial agent (and this is administered in such way as to keep all the cells in their original location) with the number of resistant colonies arising in parallel plates in which microcolonies had been spread just before addition of the agent. Clones of resistant cells would thus be spread over the plate, and while each clone would give rise no more than a resistant colony in the «unspread» plates, its members would all give rise to independent resistant colonies in the «spread» rise to no more than a resistant colony in the «unspread» plates, its members would all give rise to independent resistant colonies in the «spread» plates. Assuming equal size of the sensitive microcolonies, and calling: r = No. of microcolonies per plate containing at least one resistant mutant, i.e. equal to No. of resistant colonies per «unspread» plate; n = Total No. of microcolonies per plate; m = Mean number of mutations per microcolony; the value of m could be calculated from $\frac{r}{n} = 1 - e^{-m}$ giving $m = -\ln\left(1 - \frac{r}{n}\right)$ This is approximately $m = \frac{r}{n}$ when r/n is small. In such conditions, therefore, the mean number of mutations per plate would be $n\,m = r$. Thus if the total number of resistant colonies per «spread» plate is called R, the ratio R/r will give the average number of individuals per clone. It must be higher than unity if models 1 A or B are correct.

In Newcombe's experiment the antibacterial agent used was phage,

which was nebulized on the preincubated plates, both spread and unspread. Bornschein *et al.* tested chloramphenicol resistance in *E. coli* by this method, using membrane filters as a tool for transfer of the bacterial inoculum from the preincubation plate to the drug-agar-plate (1951). Both experiments yielded high ratios R/r, qualitatively at least in agreement with the theory of genetic adaptation. This result does not suffer from the objections raised to the fluctuation test, though it may be claimed that model 2 A is not excluded by this criterion if it is assumed that in «spread» plates, where cells are not packed in microcolonies as in «unspread» plates, resistance may arise more easily.

3) Another approach makes use of the secondary differences between independent mutations for resistance to the same drug which can be brought under observation by further analysis of the resistant colonies. Such differences, no matter whether of a morphological, biochemical, genetical character, or simply of degree of resistance should be found, if resistants appear in clones, only between members of different clones and not between members of the same clone.

Since resistants from parallel cultures will have arisen from independent mutations, among which some difference may be detected, while resistants from the same culture are likely to be members of the same clone, the comparison of variability for some suitable character between resistants from independent cultures with that between resistants from the same culture will permit a test of the clonal origin of mutants, under the form of a *test of the correlation between relatives*. If the character tested is quantitative in nature, (e.g., degree of resistance) the test will take the statistical form of a study of intraclass correlation and thus may be performed as a straight analysis of variance by comparing the variance between cultures with that within cultures. This method has been applied to chloramphenicol-resistance in *E. coli* (Cavalli 1952) where a high correlation between relatives was found using degree of resistance as the differential character. The same approach was used by Mitchison, and pursued further by him in the course of study of streptomycin-resistance in *E. coli* (1953); here, using various differential characters it was possible to recognize clones within cultures and show that the number of clones was distributed at random among the various cultures.

This test is exposed to the objection of confusion between environmental and genetic variability in a way somewhat different from that to which the fluctuation test is subjected, if it is assumed that uncontrollable environmental variation between cultures may favor one or other type, form or degree of resistance, varying with the culture.

4) Although we may now be asking too much of the potentialities of environmental variation if we want to explain all the above results by model 2 A plus environmental variation between cultures, and to consider the agreement with the genetical model as purely coincidental, a *test of the environmental variation* may be useful at this point. Such tests can be devised along lines such as those tried for chloramphenicol-resistance in *E. coli*, in which both the number of resistant colonies and the average resistance of a sample of them from each of a series of parallel cultures were tested and the parallel cultures were found to differ significantly both for number of resistants (fluctuation test) and for average resistance (correlation between relatives). If such differences were due to environmental variation between cultures, it would be expected that factors favoring the appearance of a high number of mutants in one culture should also increase their average degree of resistance and hence these two variables should be correlated. Instead a correlation of zero was found, (Cavalli 1952), showing no evidence of environmental variation. This example encourages one to believe that variation of environmental origin between parallel cultures may be negligible, although tests for this source of error should be provided whenever possible.

5) A technical device known as replica-plating permits one to show the clonal origin of resistant mutants in a more direct way than any of the above methods. This method also allows one to obtain the second kind of proof postulated above, namely the establishment of resistant strains in the absence of the drug (by sib-selection). It will therefore be described under the following section.

INDIRECT SELECTION.

Let us consider, hypothetically, the types of experiment that are capable of separating the environmental and genetical components of variance. To do this, only a simple extension of the design of the fluctuation experiments is required. Let us, as before, consider groups of cultures, prepared from small inocula, in a uniform medium. The initial series will be considered as a parental group, whose descendants will be compared in order to demonstrate the hereditary component of variance. For simplicity, let us consider only the two extreme cultures of the parental series, those from which samples give the highest and lowest assays, respectively. It has been urged that such extremes, instead of being the expected result of pre-adaptive mutation and clonal growth of resistant cells, in the absence of any drug, may instead reflect uncontrolled physiological differences between the cultures, which influence

the likelihood of an adaptive response to exposure to the drug (Dean and Hinshelwood, 1952). On this physiological hypothesis, a second cycle of growth must be expected to smoothe out any transient differences in inocula taken from the extreme cultures, so that whatever their own fluctuation, «high» and «low» filial series should show no correlation with their parents in the average assay of resistant cells. On the genetic hypothesis, however, the result will depend on the size of the inoculum which is taken from the parental «high» and «low» respectively, and distributed to the two series of filial cultures. In particular, the filial high series should again display the same high average assay, and with relatively little further fluctuation, provided each sample inoculated was large enough to contain at least one resistant (as calculated from the assay of the parental high). On the other hand, if the inoculum sample is made so small as to exclude the likelihood of introducing a resistant cell into the filial culture, the genetic component of variance could not be propagated, and the parental and two filial series should show the same average assays, with a high variance from culture to culture. In principle (though this would be quite tedious in fact) one could adjust the inoculum size so that, on the genetic hypothesis, a resistant cell is inoculated into only an occasional filial tube, which is then detected by its high assay. Repetition of this procedure would accomplish a gradual concentration of the resistant cells, relative to the sensitive, and ultimately a purely indirect selection and purification of the resistant mutant. To our knowledge, these experiments have not yet been executed in just this fashion, although the design should provide a definite test, without serious departures in the program of experimentation, of the criticisms of the genetic interpretation of fluctuation analysis. These hypothetical experiments are discussed here in order to introduce a radically different approach which is, however, based on the same theoretical foundation for the separation of environmental and genetic variability. The hypothetical experiments involving the tedious manipulations of many individual tube cultures can be executed much more expeditiously on agar plates by means of a technical artifice, replica plating.

Replica plating.

The most serious limitation to the treatment of these problems obviously concerns the purely technical difficulty of handling very large numbers of cells and families under adequate control. By previous methods, the clones of resistant bacteria have been identifiable only as statistical entities, or else by the destruction of their preponderant sensitive neighbours. The technique of replica plating greatly mitigates this

difficulty. Samples from innumerable clones, immobilized on an agar surface, can be trasferred or replicated to a series of copy plates without seriously disturbing their spatial arrangement. This is accomplished by using a sheet of velveteen, with its dense pile of vertical fibers as the vector for the inoculation. In practise, the initial agar plate, with its pattern of surface bacterial growth, is inverted with slight pressure onto a sheet of velveteen, mounted on a cylindrical support. The initial plate is then removed, and replaced by a series of fresh plates. Each of these will pick up an impression of bacteria from the velvet which subsequently develop into a remarkably faithful rendition of the original pattern of bacterial growth.

The accurate replication of this pattern is most obvious when it consists of discrete colonies, but the principle applies equally well when the growth on the initial plate is confluent owing to the character of its inoculum. The original plate is left sufficiently undisturbed by taking a velvet replica that it can be used repeatedly for the same or other purposes.

Two adaptive responses have been studied in detail by means of replica plating technique, resistance to streptomycin and resistance to the phage T_1 in Escherichia coli strain K-12 (Lederberg & Lederberg, 1952), with concordant results. Plain nutrient agar plates were spread with several thousands of sensitive bacteria, so that a smooth, confluent growth developed after 24 hours incubation, while no resistants were included in the inoculum. A series of replicas was then made to plates of streptomycin agar. In order to ensure objective registration of the plates, pins were included in the support blocks to mark the agar surfaces. After 24 hours, resistant colonies would often develop on the replica plates. More than half of these were observed to occupy superimposable positions on the successive replicas! This could be explained only by the derivation of these resistant colonies from localized clusters of cells on the original plain agar plate. These clusters are, of course, the clones subsumed by genetic theory; the physiological theory can offer no plausible explanation for such clusters in a uniform film of growth. Rather than argue this point more fully, however, we proceed to the next and more conclusive stage of the experiments.

It will be recalled, and must be emphasized here, that the original plate has not been damaged, except for the removal of a sample of its growth, nor has it had any knowledge of the streptomycin to which only the sample on the velvet has been exposed. Nevertheless, the location of many resistant clones has been revealed by the congruent patterns of colony development on the replicas on streptomycin-agar. Let us

now assume that the conditions of growth on the original plate have been adjusted so as to permit the development of just one clone per plate, on the average. Let us assume also that the registration of the plates and the technique of transferring inocula permits us to pick an area one-hundredth of the total film of growth (i. e. about 60 mm^2) and still include the entire resistant clone. It is evident that by thus retaining all of the resistant cells on a plate, and discarding 99% of the sensitives we could accomplish a 100-fold enrichment for the resistant relative to the sensitive bacteria (provided of course that, as on the genetic hypothesis, these bacteria are actually present prior to treatment with any drug). This is, in fact, precisely what was observed, as told from the assays of cultures grown from such indirectly selected inocula. The practical enrichment factor is actually closer to 1 : 1000 (6 mm^2) than 1 : 100, but this is counterbalanced by the difficulty of controlling conditions to permit just one detectable clone per plate. However, the advantage of this procedure and one which obviates the necessity for quoting any numerical details is that it can be reiterated, by plating appropriate dilutions of the first enrichment culture, locating the resistant clones by replica plating, and picking the corresponding sites for a further cycle of enrichment. It is evident that even with the very low incidence of approximately one streptomycin-resistant cell per billion bacteria, it requires (and actually did require) only three cycles of indirect selection to increase the proportion to one per thousand. At this point, the inoculum for the next stage may be diluted to the point where discrete colonies will appear on the plain agar, and yet one or more of them will be resistant. This colony is quickly identified by a final round of replica plating and having been located, yields to conventional methods of purification. In this way, streptomycin and virus-resistant cultures, respectively, were isolated by purely indirect selection. The replica plating technique was also put to work to facilitate tests of the unifermity and stability of these resistant cultures after they were once purified.

The one detail of the foregoing account that must be kept in mind is that the indirectly selected bacteria (or rather their ancestors) have never been exposed to the drug. The drug was used exclusively to locate the resistant clones by tests of their sibs, transferred via velvet. In fact, the manipulations to which the selection line has been exposed consist only of several cycles of plating on plain agar, the removal of a sample by a sheet of velveteen, and after an appropriate site has been revealed by comparison of the replica plates, picking with a platinum loop to plain

broth. The same manipulations lead, in one case to a streptomycin-resistant pure culture, in the other to a virus-resistant, depending on the auxiliary procedures which do not directly affect the main selection line. There is no opportunity whatsoever for any chemical reaction of the drug to play a role in the establishment of the resistant mutant. We do not address ourselves to the question whether individual resistant bacteria may not in fact be only uniformly but potentially resistant until they actually encounter the drug. We note, however, that each cell of the indirectly selected culture promptly develops into a colony on the streptomycin agar, which destroys all but a minute fraction of the parental culture. In addition, this potential is inherited for an indefinite number of generations to all of the progeny, prior to any exposure of these cells to the drug. When taken together with the evidence of clonal occurrence from the first stage of the experiment, the success of indirect selection provides incontrovertible evidence for the genetic theory for those systems to which it has been applied.

As in previous sections, we must give some consideration to the possibilities of a spurious negative result. The resistant mutant must not be drastically selected against on plain agar -- we have no hope, for example, of isolating a streptomycin dependent mutant by this method. It is also essential that single resistant cells from the initial plate (assuming the genetic hypothesis) be unequivocally identifiable on the selective replica plate, and these not be confused with mutants that might ultimately develop from populations growing on the latter, where selection is insufficiently drastic. If indirect selection should fail when applied to an untreated population, but succeed in extracting added resistant bacteria, originally isolated by direct selection, it would be necessary to concede that direct selection is relevant to the development of resistance. Among the most obvious possibilities that would have to be excluded by further analysis is sequential selection, in two stages, for cases when only the first step of resistance is likely to be present as such in unselected population. However, the positive results already obtained are quite unambiguous, and further discussion of spurious negative results may be deferred until specific details are submitted for adjudication.

Drug-resistant variants have also been secured in the absence of the drug by selection for correlated characters (English & Mc Coy, 1951. Gale, 1949; Goldstein, 1952). This approach also confirms the notion of preadaptation, but is not a rigorous proof of it, as the unusual physiological conditions could be regarded as the specific inciters.

III. - DIRECT GENETIC EFFECTS OF DRUGS

The proven role of mutation and selection in the development of drug-resistance in bacteria, and the vague contrary evidence that has been offered serve only to accentuate interest in a few clearcut cases of specific direct genetic effects of antibacterial agents on microorganisms. We do not refer here to the possibility that some antibiotics may have some incidental mutagenic activity of a nonspecific character (Newcombe & Mc Gregor, 1951). Streptomycin exerts a most remarkable influence on the unicellular alga, *Euglena gracilis* (Provasoli, Hutner and Pintner, 1951; cf. Pringsheim, 1952) as well as on some higher plants. Euglena cultures exposed to streptomycin suffer a rapid and irreversible bleaching, which results from the destruction of the chloroplasts. Fortunately, Euglena can be cultured heterotrophically, but the apochlorosis is permanent, and the chloroplasts are never restored. The organisms which have been found to respond to streptomycin in this way are very limited in number, perhaps for incidental reasons, and none are quite suitable for genetic analysis, so that it cannot be asserted where the streptomycin acts. It seems most likely however that this is a direct destruction of the chloroplasts, which have been believed to possess considerable genetic autonomy from other (not entirely conclusive) experiments. In a sense, the apochlorosis is a result of chemotherapy, the «curing» of the plant of its plastids (see discussion in Lederberg, 1952). In somewhat analogous fashion, the exposure of yeast to acriflavine induces the development of small colony variants, which have evidently lost part of their (mitochondrial?) respiratory apparatus. In this case, morphological analysis is incapable of demonstrating the direct locus of effect of the acriflavine, which may be more complex than the simple destruction of the target organelle, (Ephrussi, 1951). The end effect, like that of streptomycin, is believed to be loss of a genetically autonomous particle, in this case population analyses have confirmed that the chemical effects are in fact inductive, and not selective. This has been confirmed for the yeast by beautiful single cell experiments, in which individual buds engendered in the presence of acriflavine were shown to develop almost consistently into the small colony variant. A third example concerns the parabasal body of hemoflagellates; long known to disappear during the development of chemotherapeutic resistance to trypaflavine. Circumstantial evidence favors the conclusion that the aparabasal variants are induced by the drug, perhaps by interference with the division of the parabasal granule during cell fission (Piekarski, 1949), but the problem has not been closely studied from a genetic viewpoint. There is no evidence in any of these

examples that the induced variant enjoys any selective advantage over the normal wild type, a circumstance that contributes to the clarity of the proof that they are induced. It is therefore doubtful whether these variants can be regarded as adaptive or drug-resistant, and their discussion is therefore not directly pertinent to our paper.

A final example lies on the semantically confused boundary of parasitology and genetics (Lederberg, 1952). Kappa, a determinant of the killer trait in *Paramecium aurelia* shares many attributes of a cytoplasmic hereditary determinant on the one hand, and of an intracellular symbiotic microorganism on the other (Sonneborn, 1950). It has been found that kappa is destroyed by cultivating paramecia carrying them in the presence of chloromycetin or of purine analogues (Brown, 1950; Jacobson, 1952). This is no more decisive for the « genetic quality » of kappa, than is the effect of streptomycin for the chloroplasts. The four examples share the principle that an apparently autonomous, but somehow dispensable, hereditary particle is lost under the influence of the drug, and illustrate a surprising application of chemotherapeutic principles to the problems of genetics. The importance of such cases provides all the more reason why possible examples in the bacteria should be supported by ample proof. It is difficult to quote any at the present time which are not beclouded by the undisqualified possibilities of mutative, selected change. Three which perhaps deserve closer attention are the attenuation of virulence of the crown-gall bacterium in the presence of amino acids (van Lanen et al., 1952); the development of small colony variants of *E. coli* in the presence of naphthaquinone and other chemicals (Colwell, 1946) and antigenic changes in streptococci grown in the absence of acetate (McIlroy, Axelrod & Mellon, 1948).

For want of more information, Voureka's findings may also be summarized at this point. Her first finding was that a penicillin resistant strain of *Staphylococcus*, became sensitive when exposed to DNA extracts of sensitive bacteria. At the time, this was thought to be possibly analogous to the pneumococcus transformation. It was later found, however, that extracts of the resistant bacteria had the same effect. Most recently, Voureka (1952) has reported a single cell analysis of staphylococci exposed to various injurious agents such as chloramphenicol, penicillin, nitrogen mustard, and hydrogen peroxide, each of which seems to induce the same syndrome, which includes increased sensitivity to penicillin and streptomycin, and a loss of coagulase formation, mannitol fermentation, and virulence. The mechanism of these changes is obscure, but in view of the non-specificity of the inciters is clearly not adaptive. Voureka notes that the cultures have also lost their phage-

specificity and this, together with some of the circumstances noted for the development of colonies from single treated cells suggests that the phenomenon may be related to the induction of lysis by latent bacteriophage (Lwoff, 1952). The report of morphological changes induced by treatment with chloramphenicol + antiserum was confirmed by Manten and Rowley working on *E. coli* K-12 (1953). Again these changes had no adaptive value.

IV. - THE ROLE OF PHYSIOLOGICAL ADAPTATION

The experiments which were referred to in section II give strong experimental support to the belief that genetic adaptation plays a major role in the origin of drug resistance. They were not, however, designed to test effects predicted by schemes of the kind postulated under 2B, because they deal essentially with cases where only a small minority of cells survive the action of the drug or at least develop, in its presence, into a visible colony, which has acquired some stable degree of resistance as shown by subculturing in the absence of the drug. Such experiments can discriminate between models 1 (A or B) and 2 A, in the sense that the first two give rise to a precise hypothesis which can be accurately tested, and which has not been contradicted by any of the available, critically collected data. Complete exclusion of model 2A would demand a perfect fit of model 1 and for this more work may be necessary, at least for some systems. It is of importance, however, that none of the analyses sofar reported are capable of throwing doubt on the existence of mechanisms of type 1.

Model 2 A may arise either as a consequence of direct induction of the genetic change by the drug, or as an extension of model 2B under consideration of simultaneous partial killing. Model 2B has the advantage that it can be more easily put to a straight experimental test than model 2A. It is a classical basis for the description of enzyme adaptation to carbon sources, it should be noted, however, that in all cases analysed the rate of deadaptation would seem to be of the same order of magnitude as the adaptation rate. A brilliant experimental confirmation that cells adapt has been recently given by Benzer (1953) for the adaptive synthesis of beta galactosidase in *E. coli* ML. Experiments showing that this is possible for drug resistance are scanty. Baskett (1952) was able to increase resistance to proflavine in *B. lactis aerogenes* by adding repeatedly small sublethal amounts of the drug to a growing culture. Cells could thus be induced to grow at a drug concentration which, when applied to unprimed cultures would cause a killing of

$1 - 10^{-6}$) cells, i. e. a level at which one may hope to select some resistant mutants, if any were available. Adaptation was rapidly lost in subculture on drug free medium. This could be a good example of standard physiological adaptation.

Eagle, Fleischman and Levy (1952) found somewhat more stable changes in resistance by selecting survivors in plates containing sublethal concentrations, i.e. just above the threshold for some observable inhibitory action. The change in resistance of the survivors seemed to be correlated with the level of drug used for selection. Heritability of such changes should be tested before conclusions on their nature can be reached. This result is, incidentally, not entirely in agreement with observations on similar lines by Hughes (1952), Demerec (1945), Newcombe and Hawirko (1949).

It may be useful to note, at this point, that when incompletely bacteriostatic drugs, or drug concentrations, are employed, the applicability of model 2B may be erroneously claimed. Subvisible growth of the cells seeded may result in an actual input of sensitive cells far higher than that assumed on the basis of the inoculum employed.

One clear cut case of adaptive response to drugs is penicillinase formation in *B. cereus* (Pollock). In this case, it is obvious that addition of subminimal amounts of penicillin would protect a culture from the later addition of greater amounts of the drug. Knox and Collard were able to show that growth at higher temperatures (42°) which blocks penicillinase adaptation is far more easily inhibited by penicillin than growth at a temperature permitting adaptation (at 37°); preadaptation of the culture greatly increased resistance at 42°.

On the basis of the early work on drug-resistance in Paramecia it has also been claimed that stable changes in resistance without the intervention of nuclear changes are possible (*Dauermodifikationen*). The recent work on induced antigenic changes in *Paramecium aurelia* (Sonneborn, Beale), and other Protozoa (for a review, Harrison) is in formal agreement with model 2B, although here the change induced environmentally, and cytoplasmic in nature, is not, or not clearly, adaptive. On the other hand, extrapolation to bacteria of the conclusions reached in Paramecium may be dangerous. The limited knowledge available for both types of organisms indicates differences in the immediacy of nuclear control over cytoplasmic activity. It would not be too surprising if the stability of cytoplasmic systems and the latitude of variation under environmental influence and outside nuclear control were, on average, different in the two categories of unicellular organisms, though the present evidence is insufficient to warrant such a generalization.

Summing up this scanty evidence, *bona fide* physiological adaptation to drugs can hardly be expected to be missing, althoug convincing evidence that physiological adaptation of a high degree can be reached is lacking. Conditions usually employed for selecting resistant mutants, as for instance high drug concentrations with large inocula are of course of the worst type both for detecting physiological variation, and for allowing it to play freely. However one finds, especially at lower drug concentrations, a spread of individual variation in the ability to grow visible colonies, the range of this variation depending on the particular drugs and organisms employed. Part of this variation may be stochastic, i. e. the variation in the total amount of residual growth of a cell in the presence of a drug may be due to random events of a physiological nature; part may be due to the variation in the initial physiological conditions of genetically homogeneous cells; part to small genetic differences, which are not easily measured. Two facts should be stressed: 1) that no genetical theory could reject the effects of environmental variation, or previous history of the cells (as in penicillinase adaptation); 2) that the existence of physiological variation, both immediate and « potential » (adaptive) does not conflict with the fact that high and stable levels of resistance can be reached, and have been proved to be reached, by mutation and selection.

V. - THE ANALYSIS OF GENETIC INTERACTION BETWEEN INDIVIDUALS

Genetic transfers from one individual to another, i.e. the genetical consequences of sexuality in higher organisms, has been clearly demonstrated in a variety of microorganisms. While in Fungi and Protozoa, in which strains with a sexual cycle are available, the process parallels closely sexuality of higher organisms, in several bacterial species a new kind of genetical interaction has been disclosed, in which one strain can effect genetic transfers to another (with a relatively low frequency, but at any rate higher than spontaneous mutation in the receiver) without direct interaction between the cells of the donor and receiver. Any character difference tested is transmissible, although as far as present experience goes, the transfer seems to affect only one genetic character at time. This kind of transfer has been named *transduction* (Lederberg, 1952). In *Salmonella typhimurium* the agent of transfer, which acts purely as a passive carrier, has been found to be a phage. The term transduction is also meant to include an earlier described type of genetic interaction, carrying traditionally the name of *transformation*, which

has reproducibly been obtained in two bacterial species, *Pneumococcus* (Griffith 1928; Avery, McLeod, McCarthy 1943) and *Hemophilus* (Alexander and Leidy 1953). In this particular type of transduction no special carrier appears to be involved other than what is believed to be the specific inducer of the genetic change, i. e. a highly polymerised DNA.

Another type of genetic transfer has been demonstrated in bacteria, and specifically in *E. coli* (Lederberg and Tatum, 1946): namely *recombination*. Here direct interaction between the cells of the two strains is necessary. Both transduction and recombination occur as a rule with low frequency, so that the progeny, i.e. the result of the interaction must usually be selected from a mass of unchanged parents. The main formal difference between transduction and recombination is that, while in transduction only the character specifically selected for is recovered, in combination the transfer of the selected character is usually accompanied by the simultaneous transfer of other unselected markers according to a complex, but well reproducible pattern of reciprocal linkages. At present the genetic system of the more completely analysed strain of E. coli is best interpreted as formed by two chromosomes, recombination being the result of mating between compatible cells, followed by a polarised segregation which tends to eliminate preferentially segments from one mating type. Heterozygous individuals segregating for various markers have been recovered, as well as apparently fully diploid individuals. The diploid state is usually unstable, and the normal condition of the cell is believed to be haploid (though often multinucleate). (Lederberg et al., 1952; Cavalli, Lederberg and Lederberg, 1953).

While the type of analysis permitted by recombination in *E. coli* K-12 closely approximates mendelian analysis in higher organisms, transduction offers opportunities for another, more restricted type of genetic analysis which may, however, show certain advantages over the more strictly mendelian one.

Mendelian analysis.

It is important to distinguish between what may be expected, from a mendelian analysis, of drug-resistances which show the streptomycin pattern, and those showing the penicillin pattern. Or, more exactly, the distinction must be drawn between inheritance of first-step-resistance (if truly such) and that of multi-step-resistance (for drugs to which gradual adaptation is the rule). In the first instance we expect, if the theory of genetic adaptation is correct, that the genetic change to resistance is inherited as an all-or-none character, i.e. the progeny of first-step-resistants crossed with sensitive should give rise only to sensitives and resist-

ants, the progeny rembling closely either one or the other parent in respect of drug-resistance. Crosses of resistant × resistant will give rise entirely to resistant progeny, unless of course the changes in resistance represented in the two parents have originated through independent mutation at different loci, i.e. the two changes are not allelic. In the case of multistep-resistance, successive adaptation must have been due to the accumulation of successive mutations, presumably at a number of different loci. This polygenic picture was early advanced by Demerec to explain penicillin resistance in staphilococci; the later availability of crossing techniques in *E. coli* allowed one to test this hypothesis in the latter organism. At least five cases of drug-resistances have been analysed in some detail, two of them following the streptomycin pattern and three the penicillin pattern.

Streptomycin. — Various types of changes to streptomycin-resistance (or dependence) are possible in *E. coli*, all appearing as first-step mutations. Very low degrees of resistance, which are considered adaptive by Gibson and Gibson, have not been followed genetically, except in an incidental observation by Newcombe and Nyholm, on a fully resistant-strain which was presumably a second-step mutant. Differences are detectable between strains endowed with high resistance, and Mitchison (1953) distinguished four types on the basis of growth rate, stability and behaviour at high concentrations. Demerec (1950 a, b) found a variety of changes mostly of a nutritional type accompanying resistance (Sr) and dependence (Sd). It may be noted that changes correlated with the character primarily selected may be accidental, i. e. due to concomitant mutation; in which case primary and secondary character might be separated by recombination. Unless such an analysis is available, the estimation of the number of different alleles or loci cannot be considered as valid, but is likely to be excessive. Smith, Oginski and Umbreit (1949) reported that Sr mutants showed several physiological deviations, including a failure to show improvement of growth under aerobic conditions. As this is reminiscent of the small-colony variants of yeast, this effect was looked for in our own Sr cultures. All of the Sr mutants which were examined, in our own stocks as well as in the initial culture of the cited authors have displayed a typical response to aeration (Lederberg, unpublished). It is possible that the non-aerobic behaviour of the Smith-Oginski-Umbreit culture resulted from a coincidental mutation. The pyruvic acid metabolism of our Sr strains, a defect of which is claimed to be a more consistent concomitant of streptomycin-resistance has not been studied.

Mendelian analysis of changes to full resistance, performed by se-

veral authors (Newcombe and Nyholm 1950, Demerec, 1950, 1951, etc.); showed: 1) clear cut segregation into sensitives and resistants in the cross sensitive × resistant; the frequency of segregation, which depends on the mating-type of the parents (polarity of the cross) and on linkage of the resistance markers with the markers used for selecting recombinants need not concern us here; 2) perfect reversal of the ratio sensitive/resistant in the progeny of crosses in which the resistance marker is switched over from one parent to the other, keeping polarity unaltered (Newcombe and Nyholm); this is an important requirement of the mendelian theory; 3) allelism of all the various Sr mutations tested (Demerec); 4) allelism of Sr and Sd, although some room for pseudoallelism may still remain (Newcombe and Nyholm, Demerec); 5) linkage with the methionine marker, which has recently been confirmed inspite of the difficulties met in mapping this locus (Fried and Lederberg, 1952); 6) streptomycin resistance is completely recessive to sensitivity (Lederberg, 1951); attempts to secure heterozygotes of Sd with Ss or Sr have failed, perhaps because of physiological incompatibility between these alleles.

The mutation rate to streptomycin resistance has been determined at about 10^{-10} per bacterial generation in a number of bacterial strains and species. H. P. Treffers (personal communication) observed that a biotin-phenylalanine requiring mutant of E. coli K-12 displayed a much higher rate of mutation to Sr (about 10^{-6} per generation). Crossing experiments have shown that 1) this high rate is unrelated to the nutritional mutations in the stock; 2) the mutation occurs at the same S-locus; 3) the high rate is governed by a mutability modifier, separable from the S locus by recombination (P. D. Skaar and Lederberg, unpublished). A more detailed study of these effects is being made in Prof. Treffer's laboratory.

Azide. — *E. coli* K-12 can mutate in a single step to a small degree of resistance to azide. This mutation was used by Lederberg (1950) as a selective marker for recombination, in association with streptomycin. Mutation-rate to azide resistance (about 10^{-7}) is fairly high, and the efficiency of this crossing technique can be increased by using T_1 phage resistance in coupling with Az^r; the Sr parent can thus be fully eliminated (Cavalli, unpublished). This association was suggested by the close linkage of Az^r and T_1^r, Az^r having been mapped between T_1^r and the threonine- leucine markers. Only one Az^r locus has been found; also here, the switching over of Az^r from one parent to the other, keeping polarity unaltered gives results in agreement with mendelian expectations (Cavalli, 1952).

Chloramphenicol. — Resistance to this drug in *E. coli* K-12, obtained through gradual adaptation was analysed with mendelian techniques by

Cavalli and Maccacaro (1951, 1952). Crossing first-step resistants to sensitives gave a segregation into sensitives and resistants, without intermediate degrees of resistance in the progeny. Crossing first-step-resistants between themselves resulted practically always in the reappearance of a proportion of sensitives in the progeny, showing that first-step resistance is here due to mutation at a number of different loci four or five at least). Some crosser between first-steps gave rise to progeny which showed resistance higher than that of either parent, i.e. recombination mediated the accumulation of the resistance acquired by independent mutations at different loci. In a more fully analysed cross of this kind, doubly resistant mutants arose in approximately the expected proportion, calculated from the proportion of resistants in the crosses of the parental first-steps to the original sensitives. Crossing a second step to a sensitive, intermediate resistance between that of either parent appeared. On crossing multi-step resistants, obtained by prolonged « training » in liquid medium, with sensitives, the progeny was found to exhibit degrees of resistance varying over almost the full range from full sensitivity to the full resistance of the resistant parent. Linkage of resistance with the ordinary chromosome markers could be detected in this progeny, as would be expected if several loci were involved. Although there was evidence for at least some activity in a number of chromosome regions, the maximum concentration of genetic activity was near Lac. Crossing multi-step-resistants between themselves did not show positive interactions in the sense of increased resistance above that of either parent, perhaps because the physiological limit to resistance had already been reached by selection. There was instead a spread towards the low values of resistance, and some fully sensitive individuals were recovered among this progeny. This shows 1) that full or at least high resistance can be acquired in gradual adaptation through several independent paths; 2) that interaction between independent mutations is not necessarily additive. Indeed, after the first resistance- step, all successive steps acquired by selection may well be, in theory at least, due to modifiers, i. e. genes having no direct effect on resistance by themselves, but interacting with other loci to give augmented resistance. The possiblity of direct accumulation of independently obtained resistances has been observed, however.

Terramycin. — This drug is known to show a fairly high degree of cross-resistance to chloramphenicol. In the analysis by recombination of two strains of *E. coli* K-12 which had adapted to high concentrations of terramycin after a long « training » period, resistance of the progeny to both drugs was tested. Correlation was still apparent in the progeny.

but it was evident that while some changes had affected only, or mostly, resistance to one drug, others had affected resistance to both. This shows at once the existence of receptors within the cell which are « hit » preferentially, or exclusively, bq one or other of the two drugs, as well as of receptors « hit » by both approximately in the same way (Cavalli, 1952).

Furadroxyl (NF 67). — T. C. Nelson has analysed, in unpublished experiments, resistance to furadroxyl (5-nitro-2-furaldehyde-2-(2 hydroxyethyl)-semicarbazone) developed by *E. coli* K-12. At least four steps can be recognized: the inhibitory level for the parent is 4 μg/ml, and for successive steps 20-30, 40-50, 90-100, 160-200 μg/ml. The survival curves are remarkably sharp, the threshold covering only a few % of the dose. The mutation rates for each step are co. 10^{-5} - 10^{-6}, uncorrected for plate mutations. A satellite effect was prominent around resistant colonies. No mutants have been found to develop a two-step level of resistance in a single plating. Crosses of $Fx^s \times$ 1-step F_x^r showed a linkage of this particular factor to TL, with a sharp segregation into the parental categories. Different first-step mutants when crossed, yielded occasional sensitive as well as two-stepw level recombinants, along with the parental types. It is apparent that multistep resistance is here also controlled by a polygenic system. The incidence of recombination is so low, however, as to sunggest very close linkage of some of the factors.

Other drugs have been analysed to a smaller extent. Nitrogen-mustard resistance in *E. Coli* K-12 is not a single-step-resistance as in *E. coli* B, and is not correlated with UV or X-ray resistance; it shows an apparently polygenic behaviour on crossing (Cavalli, unpublished), but the instability of the drug prevents a detailed analysis. Valine has a marked antibacterial effect on *E. coli* K-12 when tested in minimal medium but none in nutrient agar, due to antagonism with isoleucine (Tatum, unpublished; Rowley, 1953). In minimal medium, strains endowed with a fairly high degree of resistance can be isolated in one step and, on crossing show linkage with M (Cavalli and Bolzoni, unpublished). This marker can be used to select recombinants in minimal.

Colicine- resistance has been analysed genetically by Fredericq and Betz. Resistance to eight types of colicines was shown to segregate in a mendelian fashion. Some discrepancy is observed in the segregations given by reversed crosses, probably due to viability differences. One of the colicines studied, K, showed cross-resistance with phage T_6, and the resistant locus showed, on mapping, to fall in a position comparable to that ascertained for phage T_6 resistance by Lederberg (1947).

TRANSDUCTION ANALYSIS.

In *Salmonella typhimurium* Lederberg and Zinder (1952) obtained the transfer of a number of genetic markers, ranging from nutritional deficiencies of various types and sugar fermentations, to drug-resistance; the only one tested here has been streptomycin-resistance. As no more than one character at a time can be transmitted in these conditions, proof of the transfer is given by the fact that the change to, say, streptomycin-resistance is significantly higher than in untreated controls. For streptomycin resistance, transduction shows an increase of 100-1000 times in the number of resistant colonies as compared with the number due to spontaneous mutation in the untreated, sensitive controls. The transfer can occur through a filter (which would bar any genetic exchange in *E. coli* K-12) and, when the transducing agent is purified, the carrier, i.e. phage, is recovered. Sr is here apparently a single-step-resistance, as could be inferred from the method of selecting the mutant.

In *Hemophilus influenzae*, Alexander and Leidy (1953) could obtain transformations of a kind similar to those classically described for Pneumococcus. The spontaneous mutation rate to streptomycin-resistance is stated to be ca. 10^{-11} per cell per generation, and the tolerance level is increased from 3 to 1000 μg/ml in one step. DNA extracts prepared from resistant cells were used for treating sensitive cells for 10 minutes, then DNA-ase was applied. If plating on streptomycin was carried out immediately at this stage, transformation of sensitives to resistants could be observed only with a low concentration of streptomycin (10 μg/ml); but the resistants thus obtained showed the same degree of resistance as the DNA-donor. If treated cells were incubated for two hours before plating on streptomycin, the rate of transformation was identical in plates with high and low concentration of streptomycin. A single step of resistance is therefore transduced, and some phenotypic lag is apparent in the development of resistance.

In *Pneumococcus*, Hotchkiss (1951) has recently added to the already existing data on antigenic transformations. He has shown that transformation was not limited to antigenic characters, but is possible for all the drug-resistances which were tested, namely streptomycin, penicillin and sulpha drugs. Pneumococcus transformations require a battery of supplementary conditions beside specific DNA from donor cells. Even in optimal conditions, transformation is limited to a few percent of the cells treated, the bar to complete transformation remaining unknown. Of the drugs tested, streptomycin resistance is acquired by mutation, and transmitted by transformation, in a single-step. Penicillin resistance is

of greater interest since the donor strain had acquired resistance by long training and hence by a number of steps (the number of which could not be determined exactly on the evidence of the adaptation process alone). DNA extracted from the donor strain could impart resistance to the sensitive receiver only in three successive steps: first exposure provided resistance from the initial level of 0.01 μ/ml to 0.05 μ/ml; second exposure of the first-step-transformed strain increased this level to 0.12 μ/ml, and third exposure could still raise this to 0.30 μ/ml. The polygenic picture comes out very prettily from this experiment, where the peculiarity of the « hybridisation » process (one factor transduced at time) seems to split the complex polygenic system developed by selection into its elementary components. It would be interesting to know if the order with which these components are transduced is that followed by selection, as would happen for instance if the changes due to later steps were only modifiers of the resistance resulting from the first-step without a direct effect on resistance. The publised data do not seem to warrant a conclusion on this point. The use of donor strains with a known number of steps, and of various independent first- or multisteps as donors or receivers might certainly add, in an interesting way, to our knowledge of the polygenic behaviour in drug resistance.

* * *

Thus both mendelian and transduction analysis confirm the view that single-or multi-step types of resistance are the result of elementary and composite changes of a discrete nature respectively, as demanded by genetic theory. They leave no room for theories demanding blending inheritance, at least at the levels at which experimentation has been carried out. One last point which may deserve mention, however, is a curious misconception evoked by the pneumococcus transformation and other types of transduction, which have been quoted as examples of directed mutation, by way of justification of drug-induced adaptations. It should be emphasized that transformations, as well as the other types of genetic interactions between individuals listed in this paragraph, are but species of genetic transfer; and hence they lend no support direct or otherwise to the theory of drug induced adaptations. (Hobby, 1953; others).

VI. - THE CONSEQUENCES OF THE GENETICAL THEORY OF DRUG ADAPTATION

The roles of mutation and selection have been sufficiently documented by the above summarised investigations to convince us that drug

adaptation is, at least to a large extent, a matter of genetical adaptation, and in this way does not differ from other kinds of adaptive changes observed in the same and other organisms. Thus bacteria do not seem to differ greatly from higher organisms in their evolutionary mechanisms, and unusual or complex hypothetical mechanisms, devised to predict the adaptive response to drugs in bacteria, may have to fall under Ockam's razor.

Although the nature of the mutation process remains fundamentally obscure, we know that it can provide a population with a great variety of heritable changes, and in this way the demands of new environments can be met in such a way that the survival of the population is guaranteed even against extreme conditions, through the survival of some individual which can both withstand and propagate themselves within the adverse environment. If the change to the new environment is permanent, or at least long-lasting as compared with the generation time of the organism, there will be opportunities for further genetic adaptation, so as to raise the level from bare survival of the population to optimal or nearly optimal conditions of growth. If the change is transient, return to the original form by back mutation is possible and if the original type had a selective advantage over the mutant in the old conditions, as is usually the case, the population will shift back to the primitive condition. The multiplicity of genetic changes effecting a similar result, however, and the rapid accumulation of secondary changes of adaptive significance will in most instances determine differences between a population before, and after a cycle of genetic adaptation to a new transient environment, followed by deadaptation (or readaptation) to the old enviroments. The change may or may not be easily detectable. If no difference is detected after a cycle of genetic adaptation and readaptation, we say that the change was unstable (and, of course, an almost unlimited range of instability may exist). When a difference is detected, which may or may not reflect a partial return to the original drug-sensitive state, we have been faced with the irreversibility of evolution.

That the variety of genetic changes available in a population is a very great one is shown by a wealth of direct or indirect information. The variability of independent first-step-resistants provides evidence of the variation that is directly available for selection to play upon. A number of apparently unrelated changes are accompanied by variation in drug resistance. Thus, when selecting for aminoacid deficient mutants, an increase in penicillin sensitivity may be observed (Plough and Grimm, 1949; Rowley, 1952); when testing individuals selected for resi-

stance to a drug, they often show changes in resistance to other drugs, both increased (cross-resistance) and decreased (induced sensitivity, Szibalski and Bryson, 1952). Resistance for chloramphenicol has occasionally been accompanied by the loss of penicillinase formation. A great variety of hereditary changes of this kind may be given, so that we can confidently expect that a population which originated from a single cell some twentl or thirty generations before may already contain a wealth of new heritable variation. It is not too surprising, therefore, if different isolates from a bacterial population show differences of sensitivity (Hughes, 1952), although minute differences in resistance may be overestimated, if careful control by suitably planned experiments is not exercised to check day-to-day variation.

If, inspite of the large supply of new variability, bacterial populations grown on a constant medium rarely show apparently spontaneous changes in behaviour, at least for most of the characters analysed, the reason can only be that most of the new variation has an adaptive value, in respect of the standard environment, inferior to that of the ordinary type. Most mutants therefore will be outgrown. This is simply the consequence of the fact that the strain has reached some degree of genetic equilibrium, i.e. it has concentrated at least the most frequent changes which are more favorable to reproduction in the standard environment. Experience shows that these equilibria are largely dictated by the equilibrium of mutation and selection, rather than by equilibrium between forward and backward mutation rates. Therefore it is not surprising that most mutants have lower growth rate than the parental form, as shown by Demerec (1950) for streptomycin-resistance.

An altered environment, such as may result from the addition of a drug, is bound to destroy this equilibrium. If the change is not too drastic, the original type may multiply to some extent, and its frequency will decrease from almost one to some value intermediate between one and zero; if the change is very drastic, it will fall down to zero. A special situation may arise if the resistant mutants may « help » the sensitive parents to survive or even multiply in the drug-medium; an extreme example being that of partial or complete destruction of the drug by the mutant, or the production of metabolites antagonistic to the drug (PAB-producing mutants in sulfa drug resistance, for instance). Very complex equilibria may be created in bacterial populations in this way, and the fact that apparently resistant colonies, obtained in selection in liquid, and even in solid drug-media may give rise to partially or fully sensitive subcultures, simulating reversion (Banic, 1953) may be explained by the partial survival and transfer of

the sensitive parent. The careful, possibly repeated isolation of single colonies from resistant mutants on drug free media, before any test of stability, is carried out on them, seems therefore an elementary (though a somewhat neglected) precaution.

When rapid training to high concentrations of drugs like chloramphenicol is carried out, the general viability of the strain, as shown by growth rate or density at saturation, etc., is usually decreased. Twenty strains of *E. coli* K-12 independently trained to high chloramphenicol-resistance showed a density at saturation between one third and one half that of the original sensitive strains, in the absence of the drug, and still smaller in its presence. It is not surprising therefore that these strains showed a tendency to revert in the absence of the drug; this would indeed be inevitable, if back mutation is possible. Return to sensitivity, if it occurs, is usually gradual; full reversion of a highly resistant strain may take six months or more of weekly transfers though it may occasionally be more rapid; sometimes complete or even partial reversion has not been observed in three of four years.

Experimental proofs have been given that return to sensitivity in these well established cases of genetic resistance is due to (back) mutation and selection. Studying artificial mixtures of sensitives and resistant mutants one finds, as a rule, that the latter are outgrown (Demerec, 1950; Welsch, 1952). It might be argued that such experiments cannot rule out transformation unless the two strains carry suitable genetic markers. Spontaneously occurring morphological changes were used by Harm (1951) to follow the fate of population of staphilococci exposed to the action of phenol and formaldehyde; mixed cultures of sensitives and resistants, marked by their colonial morphology, showed continuous changes in the proportion of the two morphological types in the sense of an increase in the frequency of the resistant type when the drug was added, and of the sensitive type when the drug was withdrawn. Experiments with artificially marked strains were performed using streptomycin resistance in *E. coli* K-12 (Cavalli, unpublished): mixtures of sensitives and resistants marked by Lac fermentation were followedw by counts on EMB Lac with and without streptomycin after every third transfer. Resistants were outgrown in six to ten transfers starting with equal frequencies. The genetic markers used (other markers were available in the strains additional to Lac) each had its own effect on the rate of the elimination. The rate of elimination due to the Ss/Sr difference only was equivalent to a difference of 14% between the growth rate of parent and mutant; analysis of the growth rate of the two strains grown independently confirmed this difference. It is of interest that in a control culture

of this experiment with only Sr individuals originally, at the 9th transfer a clear cut discrepancy between plate counts with and without streptomycin appeared: it was ascertained that only 3% of the cells were resistant, the other being fully sensitive. This culture on further transfers showed a progressive decrease of the proportion of resistants, with the same rate of elimination as a comparable artificial mixture of sensitives and resistants. Mutation to sensitivity must have occurred in this culture, a very rare event for streptomycin-resistance in *E.coli*. Reversion rates have been studied with great detail in the more favorable case of streptomycin-dependents (Bertani, 1951; Demerec, 1951) Sd can revert to both Sr and Ss with approximately equal ease, and rates which are usually low but vary from to strain.

The geneticist tends of course to deal preferentially with stable hereditary changes. Apparently resistant colonies which, for some reasons or other, very easily revert to sensitivity on subculture in the absence of the drug will obviously be discarded in view, for instance, of mendelian analysis. The objection may be raised that this will inevitably create a selection of the material analysed. This selection cannot be very important, since is is easy to secure a wealth of stable resistants for genetic analysis with most systems. On the other hand, adaptationists who dismiss or minimise the role of mutation and selection claim that stable resistants of non-mutative origin can be obtained at least by prolonged training; but, whenever such material has come under genetic analysis, it proved to contain a great deal of genetic variation. Also, the evidence from Dauermodifikationen in Paramecium shows that environment-induced changes of extranuclear origin may be stable; so that the use of stable resistants for analysis should not discard all extranuclear changes, if these actually exist in bacteria. It may be added that relative instability should be checked, at any rate, with frequent single colony isolation and control of resistance of subcultures of isolates without re-exposure to the drug of the strain, to avoid the selection of other changes.

The problem of considering the origin of unstable variants still remains. Full rapid reversion, demanding, on a genetic interpretation, a hypothetically high back mutation rate, would certainly be more easily interpreted as due to physiological adaptation, and is at any rate difficult to submit to experimental analysis. Such rapid reversions however seem more frequent only with low degrees of resistance, at the fringes of physiological variation, or in tests for physiological adaptation designed to exclude the intervention of selection.

The relative importance of genetic and physiological adaptation in the development of drug-resistance of bacteria seems therefore to be in

favor of the genetic mechanism. The reverse may well be true with Protozoa or at least some Protozoa; it would indeed be surprising if such a delicate balance were adjusted at exactly the same level for all organisms and drugs. It is also possible of course that for some drugs, physiological adaptation may play a more important role than for others, thus leading workers who use these particular drugs to make undue generalisations on the overwhelming role of physiological adaptation.

Whatever the role of physiological adaptation, the exact limits of which are difficult to assess at present, some speculation concerning it may be of interest. It is clear that whenever physiological mechanisms can cover a wide range of adaptability, all mutants whose phenotypic effect is smaller than this range will de damped by the more efficient phenocopy which will rapidly arise through physiological adaptation, or be already present in the form of non-genetic variation. A large range of physiological variability, immediate or potential, will therefore tend to suffocate genetic adaptation. On the other hand, if this range extends only to somewhere midway between the parental average and the mutant average, physiological adaptation will smoothe the way to the genetic one, by favouring partial growth of the tail of the distribution curve and thus increasing the chances of mutation.

Drug resistance has been given a prominent place in the study of evolution of microorganisms essentially in view of its importance for chemotherapy, and, to a lesser extent, antisepsis. Some practical conclusions may therefore be appropriate. That this kind of evolution can affect the praxis of chemoterapy has been shown by the accumulation of failures of certain therapies due to the increasing frequency of drug resistant pathogens: sulfonamide resistant gonococci, penicillin resistant staphilocci, streptomycin and isoniazide resistant tubercle bacilli being but the outstanding examples.

The answer to this danger has been mostly found in the development of new drugs unrelated to the old ones; in the employment of massive doses since the beginning and without interruption, in the association of local and general antibiotic therapy, and lastly and more easily by the association of drugs. Genetics offers an easy explanation for the success of drug associations, since with drugs to which no cross-resistance (due to similarity of mechanism of action) is observed, one drug will destroy the few resistant mutants left by the other. In this connection, the importance of using simultaneously associated drugs, and not in sequence, hardly needs being stressed. *Genetic synergism*, i.e. absence of cross-resistance, is to be kept distinct from *physiological synergism* between drugs. A first foundation for a map of genetic

synergism was laid by Szibalski and Bryson, wha analysed strains made resistant to each of 15 different antibiotics for all possible cross-resistances. The number of tests necessary in such investigations is lessened by the use of a rapid technique of scoring for resistance which they have developed, namely the gradient plate technique (Bryson & Szibalski).

The interaction between two drugs at the physiological level may range from true synergism (joint effect greater than expected on simple addition of effects) to simple addition, indifferent effect, or antagonism. Physiological synergism is, to some extent, negatively correlated with genetic synergism (Klein and Schon, 1952). In fact an additive interaction may be expected between related drugs like chloramphenicol and terramycin, which show cross-resistance and therefore genetic antagonism. On the other hand unrelated drugs like terramycin and penicillin show genetic synergism and physiological antagonism (Jawetz et al., 1953; Klein and Schon, 1952) due presumably to the fact that penicillin is actively bactericidal only on growing cells, and a bacteriostatic drug would therefore greatly reduce its action. In other cases, such as the association of chloramphenicol and sulpha drugs, or for the therapy of tuberculosis, the asociation of isoniazide and streptomycin, probably both genetic and physiological synergism occur. At any rate, the rules of physiological synergism are still largely unknown, and show variations form strain to strain. Therefore, any remark we can make must, at this stage, be qualified by a number of reservations so that every case of therapy must, in the end, be judged on its own merits.

LITERATURE

Alexander H. E. & Leidy G. (1953). Induction of streptomycin resistance in sensitive Hemophilus influenzae by extracts containing desoxyribonucleic acid from resistant Hemophilus influenzae. J. exp. Med. 97, 17.

Armitage P. (1952). The statistical theory of bacterial populations subject to mutation. J. Roy. Statistic. Soc. Series B., 16, 1.

Avery O. T., Mac Leod C. M. & Mc Carty M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. exp. Med., 79, 137.

Banic S. (1953). Ueber die Hypothese von den reversen Mutationen der Bakterien. Schweiz. Ztschr. allg. Path. Bakt. 16, 112.

Baskett A. C. (1952). The resistance of Bact. lactis aerogenes to proflavine (2:8-diaminoacridine). II: The direct induction of adaptation. Proc. Roy. Soc. B., 39, 251.

Beale G. H. (1952). Antigen variation in *Paramecium aurelia* variety 1., Genetics, 37, 62.

Benzer S. (unpublished).

Bertani G. (1951). A method for detection of mutations, using streptomycin dependence in Escherichia coli. Genetics, 36, 598.

Bornschein H., Dittrich W. & Höhne G. (1951). Zur Entstehung der Chemoresistenz bei Bakterien. Naturwiss. 16, 383.

Brown C. H. (1950). Elimination of kappa particles from « killer » strains of Paramecium aurelia by treatment with chloromycetin. Nature, 166, 527.
Bryson V. & Szybalski (1952). Microbial selection. Science 116, 45.
Catlin B. W. (1951). Response of *Escherichia coli* to ferrous ions. J. Bact. 65, 413.
Cavalli L. L. & Maccacaro G. (1950). Chloromycetin-resistance in *E. coli*. A case of quantitative inheritance in Bacteria. Nature, 166, 991.
Cavalli L. L. & Maccacaro G. A. (1952). Polygenic inheritance of drug-resistance in the bacterium *Escherichia coli*. Heredity, 6, part 3, 311.
Cavalli L. L. (1952). Genetic analysis of drug-resistance. Bull World Hlth. Org., 6, 185.
Cavalli L. L., Lederberg J. & Lederberg E. M. (1953). An infective factor controlling sex-compatibility in *Bacterium coli*. J. gen. Microbiol., 8, 89.
Colwell C. A. (1946). Small colony variants of Escherichia coli. J. Bact. 52, 417.
Dean A. C. R. & Hinshelwood C. (1952). The resistance of Bact. Lactis aerogenes to proflavine (2:8-diaminoacridine). I: The applicability of the statistical fluctuation test., Proc. Roy. Soc. B., 139. 236.
Demerec M. (1945). Production of Staphylococcus strains resistant to various concentrations of Penicillin. Proc. Nat. Acad. Sci. 31, 16.
Demerec M. (1948). Origin of bacterial resistance to antibiotics. J. Bact., 56, 63.
Demerec M. (1949). Patterns of bacterial resistance to penicillin, aureomycin and streptomycin. J. Clin. Inv., 28, 891.
Demerec M. (1950a). Reaction of populations of unicellular organisms to extreme changes of environment. Amer. Naturalist, 84.
Demerec M. et al. (1950b). The gene. Carnegie Inst. Yearbook, 49, 144.
Demerec M. (1951). Studies of the streptomycin-resistance in system of mutation in E. coli. Genetics, 36, 586.
Eagle H., Fleischman R. & Levy M. (1952). Development of increased bacterial resistance to antibiotics. I: Continuous spectrum of resistance to penicillin, chloramphenicol, and streptomycin. J. Bact. 63, 623.
English A. R. & Mc Coy E. (1951). A study of streptomycin resistance in micrococcus var. Aureus. J. Bact. 61, 51.
English A. R. & Mc Coy E. (1951). Growth comparisons of streptomycin-sensitive and streptomycin-resistant Micrococcus pyogenes var. aureus. J. Bact. 62, 19.
Ephrussi B. & Hottinguer H. (1951). Cytoplasmic constituents of heredity. On an unstable cell state in yeast. Cold Spring Harbor Symp. 16, 75.
Eriksen K. R. (1949). Studies on the mode of origin of penicillin resistant staphylococci. Acta Path. et Micr. Scand. 26, 269.
Frédéricq P. & Betz-Bareau M. (1952). Recombinants génétiques de souches marquées par résistance aux colicines et aux bactériophages. Ann. Inst. Pasteur 83, 283.
Fried P. S. & Lederberg J. Linkage in *E. coli* K-12. Genetics: 37, 582.
Gale E. F. (1949). The action of penicillin on the assimilation and utilization of amino-acids by gram positive bacteria. Selective Toxicity & Antibiotics, Symp. Soc. E. B. III, 235.
Gale E. F. & Rodwell A. W. (1949). The assimilation of amino acids by bacteria. 7. The nature of resistance to penicillin in Staphylococcus aureus. J. gen. Microbiol. 3, 127.
Gibson M. I. & Gibson F. (1951). Development of resistance to dihydrostreptomycin by Bacterium coli. Nature 167, 113.
Goldstein A. (1952). Effect of penicillin on streptomycin-dependent variants in Escherichia coli populations. Science 116, 113.
Graessle O. E. & Pietrowski J. J. (1949). The in vitro effect of para-aminosalicylic acid (PAS) in preventing acquired resistance to streptomycin in Mycobacterium tuberculosis. J. Bact. 57, 459.
Griffith F. (1928). The significance of pneumococcal types. J. Hyg. 27, 113.
Harm W. (1951). Untersuchungen zur Frage der Variabilität der Formaldehyd und Phenol-Empfindlichkeit von Micrococcus pyogenes var. aureus (Staphylococcus aureus). Arch. f. Mikrobiol. 16, 319.
Harrison J. A. (1947). Antigenic variation in protozoa and bacteria. Ann. Rev. Microbiol. 19-42.
Hinshelwood C. M. (1946). The chemical kinetics of the bacterial cell. Oxford Univ. Press, 1946.
Hobby G. L. (1953). Symposium on the mode of action of antibiotics. Part. II: The mode of action of terramycin and eureomycin. Bact. Rev. 17, 29.
Hotchkiss R. D. (1951). Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. Cold Spring Harbor Symp. 16, 457.

Hughes W. H. (1952). Variation in penicillin resistance in single-cell cultures of Staphylococcus aureus. J. gen. Microbiol. 6, 175.
Jacobson W. E., Williamson M. & Stock C. C. (1952). The destructive effect of 2,6-diaminopurine on kappa of stock 51 killers, variety 4, of Paramecium aurelia. J. Exp. Zoology, 121, 505.
Jawetz E., Gunnison J. B., Bruff J. B. & Coleman W. R. (1953). Studies on antibiotic synergism and antagonism. J. Bact. 64, 29.
Klein M., & Schon, S. E. (1952). The role of bacterial resistance in antibiotic synergism and antagonism. J. Bact. 65: 454.
Knox R. & Collard P. (1952). The effect of temperature on the sensitivity of Bacillus cereus to penicillin. J. gen. Microbiol. 6, 369.
Van Lanen J. M., Baldwin I. L. & Riker A. J. (1952). Attenuation of crown gall bacteria by cultivation in medium containing glycine. J. Bact. 63, 715.
Lea D. E. & Coulson C. A. (1949). The distribution of the numbers of mutants in bacterial populations. J. Genetics, 49, 264.
Lederberg J. & Tatum E. L. (1946). Gene recombination in Escherichia coli. Nature. 158, 558
Lederberg J. (1947). Gene recombination and linked segregations in Escherichia coli. Genetics 32, 505.
Lederberg J. (1950). The selection of genetic recombination with bacterial growth inhibitors. J. Bact. 59: 211.
Lederberg J. (1951). Streptomycin resistance: a genetically recessive mutation. J. Bacter., 61, 549.
Lederberg J., Lederberg E. M., Zinder N. D. & Lively E. R. (1951). Recombination analysis of bacterial heredity. Cold Spring Harbor Symp. 16, 413.
Lederberg J. (1952). Cell genetics and hereditary symbiosis. Physiol. Rev. 32, 403.
Lederberg J. & Lederberg E. M. (1952). Replica plating and indirect selection of bacterial mutants. J Bact. 63, 399.
Leskowitz S., Fox Ch. L. jr. & Raymond S. (1952). The isolation and identification of PABA produced by staphylococci resistant to sulfonamide. J. exp. Med., 95, 247.
Linz R. (1950). Sur le mécanisme de l'action de la Streptomycine. II: La résistance à la Streptomycine. Ann. Inst. Pasteur 78, 105.
Luria S. E. & Delbrück M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28, 491.
Lwoff A. (1953). L'induction. Ann. Inst. Pasteur 84, 225.
Manten A. & Rowley D. (1953). Influence of chloramphenicol and specific antiserum on the variability of the K-12 strain of Bact. coli. Brit. J. Exper. Path., 34, 23.
Mc Ilroy A. P., Axelrod A. E. & Mellon R. R. (1948). The influence of sodium acetate upon the dissociation of a strain of hemolytic streptococcus. J. Bact. 56, 547.
Mitchison D. A. (1953). The occurrence of independent mutations to different types of streptomycin resistance in *Bacterium coli*. J. gen. Microbiol. 8, 168.
Monod J. (1912). Le rôle des inducteurs spécifiques dans la biosynthèse des enzymes. Symp. sur la biogénèse des protéines, p. 75. II Congrès Int. de Bioch., Paris.
Newcombe H. B. (1948). Delayed phenotypic expression of spontaneous mutations in Escherichia coli. Genetics, 33, 447.
Newcombe H. B. (1949). Origin of bacterial variants. Nature, 164, 150.
Newcombe H. B. & Hawirko R. (1949). Spontaneous mutations to streptomycin-resistance and dependence in *Escherichia coli*. J. Bact. 57, 565.
Newcombe H. B. & Mc Gregor J. (1951). On the nonadaptive nature of change to full streptomycin resistance in Escherichia coli. J. Bact. 62, 539.
Newcombe H. B. & Nyholm M. H. (1950). The inheritance of streptomycin-resistance and dependence in crosses of *Escherichia coli*. Genetics 35, 603.
Piekarski G. (1949). Blepharoplast und Trypaflavin-wirkung bei Trypanosoma brucei. Zbl. Bact. Abt. I, 153, 190.
Plough H. H. & Grimm M. R. (1949). Reversal to penicillin sensitivity in a cysteine requiring mutant of Salmonella. Science, 129, 173.
Pollock M. R. (1953). Penicillinase adaptation and fixation of penicillin sulphur by *Bacillus cereus* spores. J. gen. Microbiol. 8, 186.
Potter V. R. (1951). Sequential blocking of metabolic pathyays in vitro. Proc. Soc. Exp. Biol. Med., N. Y., 76, 41.
Pringsheim E. G. & Pringsheim O (1952). Experimental elimination of chromatophores and eyespot in Euglena gracilis. New Phytologist, 51, 65.

Provasoli L., Hutner S. H. & Pintner I. J. (1951). Destruction of chloroplasts by streptomycin. Cold Spring Harbor Symp. 16, 113.
Rowley D. (1953). Inhibition of E. coli strains by aminoacids. Nature, 171, 80.
Ryan F. J. (1952). Adaptation to use lactose in Escherichia coli. J. gen. Microbiol. 7, 69.
Smith P. H., Oginsky E. L. & Umbreit W. (1949). The action of streptomycin. II: The metabolic properties of resistant and dependent strains. J. Bact. 58, 761.
Sonneborn T. M. (1950). The cytiplasm in Heredity. Heredity, 4, 11.
Sonneborn T. M. (1950). Cellular transformations. Harvey Lectures, 49, 145.
Szybalski W. & Bryson V. (1952). Genetic studies on microbial cross resistance to toxic agents. I: Cross resistance of Escherichia coli to fifteen antibiotics. J. Bacter. 64, 489.
Voureka A. (1952). Induced variations in a penicillin-resistant staphylococcus. J. gen. Microbiol. 6, 352.
Welsch M. (1952). Some aspectof microbial resistance to streptomycin. Bull. Org. Mond. Santé 6, 173.
Yang Y. N. & White P. B. (1934). Rough variation in V. cholerae and its relation to resistance to cholera phage (type A). J. Path. Bact. 38, 187.
Yudkin J. (1953). Origin of acquired drug resistance in bacteria. Nature 171, 541.
Zelle M .R. (1942). Genetic constitution of host and pathogen in mouse typhoid. J. infect. Dis., 71, 131.
Zinder N. D. & Lederberg J. (1952). Genetic exchange in Salmonella typhi murium. J. Bact., 64, 679.